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PRINCIPAL INVESTIGATOR: Sambhu Bhattacharyya, Ph.D.

CONTRACTING ORGANIZATION: William Beaumont Army Medical Center
El Paso, Texas 79920-5001

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Rabbit tracheal explants, exposed to burning pine wood smoke, were cultured in a chemically defined medium with and without retinoic acid(±RA). 15-20 minute exposures led to a RA-independent degeneration of the mucociliary epithelial sheath. In 10 minute exposures tissue integrity was retained, but epithelial morphology changed from normal pseudostratified columnar to the flattened appearance typical of the squamous phenotype. Despite the dramatic shift in morphology, explants exhibited normal RA-dependent mucin gene expression characteristic of the mucociliary phenotype. Furthermore, electron micrographs showed continued presence of both secretory granules and cilia. RA(+) cultures also showed a normal pattern of adherent epithelial cells. In RA(-) cultures, however, there were prominent intercellular spaces indicating an RA dependence for maintaining adhesive contacts following smoke exposure. An 18-mer mucin antisense oligomer that suppressed mucin gene expression and secretion also unexpectedly blocked the smoke induced metaplasia in RA(+) cultures, but the sense oligomer had no effect. This study demonstrates that combined therapy of retinoids and antisense oligomer may have potential clinical implication in the treatment of people, including combat soldiers, exposed to smoke.

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Smoke, trachea, mucin, retinoic acid, mucin antisense DNA

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S. N. Bhattacharyya 11/26/97

PI - Signature Date

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5. Introduction

Acute respiratory distress, caused by inhalation of toxic products of incomplete combustion contained in the dense smoke from common fires and those by incendiary weapons under combat conditions, has been identified as one of the major factors contributing to the morbidity and mortality of patients hospitilized with fire associated injuries (1-6). Blockage of the respiratory tract by mucus and degenerating mucociliary epithelium are two of the primary pathophysiological events together with transient pulmonary edema. Shortly after smoke exposure, the irritated epithelium releases various eicosanoids including leukotriene B4 which is a principal chemotactic factor (7) leading to the influx of circulatory neutrophils, and it has been suggested that this influx of neutrophils is responsible for the pathophysiological events (8-10). This implies that damage could be prevented by inhibiting the neutrophil influx. In the present proposal, we have asked whether any of these events, in contrast, may be responses indigenous to the lung itself and thus, whether retinoic acid, which is known to be important in the development and maintenance of the differentiated state of the mucociliary epithelium and a mucin antisense oligomer, could have any beneficial effects on early events.

For several years, we at DCI, WBAMC, have been engaged in studying the effects of retinoids, different pharmacologic agents and antisense mucin oligomers on the mucin gene expression and secretion of mucins in organ as well as in isolated tracheal epithelial cells cultured in a synthetic medium. To date, we have found that retinoids are required for normal function of tracheal epithelial cells grown in a synthetic medium. Without retinoic acid, the cells neither expressed mucin message nor maintained normal cytological appearance (11-14). When retinoids were added back to the culture medium, the cells grew normally and the mucin message was expressed again. Addition of a mucin 18-mer antisense oligomer blocked the expression of mucin message as well as mucin secretion while keeping the cell profile normal (13,14). Squamous differentiation of epithelial cells which occurred in the absence of retinoids in the cultured medium was reversed by supplementing the medium with retinoic acid. Thus, the combined effect of both retinoids and mucin antisense oligomer may have clinical application in preventing respiratory problems of people, including combat soldiers, exposed to toxic substances like smoke. The present report describes some of our work on tracheal explants exposed to smoke generated from burning pine woods and the effects of those two agents,cited above, on the pathophysiology as wells as mucin gene expression and secretion in this system.

6. **Body**

Methods

Organ culture

Tracheal ring organ cultures were prepared from rabbit trachea (New Zealand white rabbit, Hazelton Research Laboratories, Denver, Pennsylvania). Tracheas were excised, trimmed, washed in minimum essential medium (MEM), and cut into rings (1-2 mm). Rings from tracheas (250 mg wet weight) were slit, suspended in minimum volume of the same medium, with epithelial layer towards the atmosphere and exposed to circulating smoke. After exposure to smoke, the rings were maintained in a serum-free and hormone-supplemented modified Eagle's / F-12 Ham's medium with and without retinoic acid, as described before (11-14). The cultures were incubated at 37°C in a humidified incubator containing air/CO_2 (19:1).

Smoke exposure

Smoke was generated by burning pine wood (40 grams) in a modified bee smoker with a bellow (N-4 size, A.I. Root company, Ohio) which was connected to a plexiglass chamber (48x28x30 cm) containing shelves for holding tissue culture plate. The chamber contained a temperature monitoring device. Smoke was circulated throughout the exposure chamber by a pump. Rabbit tracheal rings were exposed to whole smoke for different periods of time. After exposure to smoke, the tracheal cultures were maintained for 48 and 96 hours in the medium ± retinoic acid (0.1 μM) and mucin message in the explants was determined. The effect of phsphorothioate derivative of sense (1.0 μM) and antisense (1.0 μM) on the expression of mucin message and secretion in the explants grown in medium with retinoic acid (0.1 μM) was measured by adding these reagents to tracheas after exposure to smoke and continuing incubation for 96 hours.

RNA isolation and dot blot analysis

RNA was isolated from organ cultures by a single-step thiocyanate-phenol-chloroform extraction procedure (15). A direct transfer of total RNA (2-3 μg) to spots of the hybond membrane was made in a dot blot apparatus. The membrane was hybridized to the E-linked 30-mer mucin antisense oligomer according to the method in the E-link Plus oligonucleotide labeling kit (Genosys, Houston) and published elsewhere (11-14). The β-actin and type I keratin messsage from different sources were measured by the methods described before (11-14). The compositions of the sense and antisense oligomers (phosphorothioate derivatives) have been described previously (13,14, reprint included in this report). The density of the signals was measured by the Bio-Rad densitometer.

Incorporation studies

Mucin production in the tracheal cultures was measured by the incorporation of [6-^{3}H] glucosamine into the secreted materials, the methods of which have been described in details elsewhere (11). Radioactivity was determined in a Beckman counter after an aliquot of incorporated glycoproteins was suspende in 5.0 ml of ready safe solvent.

Light microscopy

The tissues for light microscopic studies were fixed in formaldehyde, placed in tissue cassettes and kept in the Tissue Tek VIP for 13 hours. The tissues were then embedded in paraffin. The embedded tissues were sectioned and stained with hematoxylin and eosin (H & E) reagents prior to examination.

Electron microscopy

The tissues prepared for electron microscopy were fixed in 3.5% (w/v) glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections of the sample were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (11-14).

Results

Smoke induced destabilization and metaplasia of the mucociliary epithelium

Explants were exposed to ambient temperature pine wood smoke for various times (5, 10, 15, 20 minutes) and then were assessed for changes in tissue histology and differentiation based on cytological and biochemical criteria.

Controls

Explants not exposed to smoke exhibited a normal pattern of tracheal tissue organization, namely, a pseudostratified columnar epithelium at the lumenal face overlying mucosal and cartilagenous layers respectively (Figure 1A). Ultrastructural features of the epithelium were also characteristic of normal tracheobronchial tissue, namely, a pattern of extensive lateral intercellular adhesion (zonula adherens), occasional desmosomal contacts, prominent cilia, microvilli, and small vesicles (Figure 2A). The large amorphous gray vesicles seen in micrographs of exposed explants (Figure 2B) were not seen in control. Mucin gene expression was low in these explants (Figure 2G,a).

5, 15, and 20 minute exposures

The histological and ultrastructural properties of the 5 minute exposed explants were indistinguishable from those described above for the controls (Figures 1A and 2A), whereas the longest exposures (15 and 20 minutes) resulted in extensive erosion of the mucociliary layer together with edema in the mucosa extending to the adventia. The remaining cells in the mucociliary epithelium were vacuolated (Figure 3), a condition indicative of cellular degeneration. These cells did not survive long enough in the culture medium to measure the changes reported here. Mucin gene expression in 5 minute exposed explants was similar to that of control, whereas none was detected in the 15 and 20 minute exposed samples, the latter to be expected given the extensive shredding of the mucociliary epithelium.

10 minute exposure

The properties of these explants were remarkable in being unrelated to either of the two extremes seen in the 5 minute vs. 15/20 minute exposures or to a mixed pattern with regions of each , i.e., patches of normal tissue admixed with regions lacking the mucociliary layer. Tissue

integrity was retained in the 10 minute exposed explants as in the controls and 5 minute exposure, but extensive regions of the epithelium changed from pseudostratified columnar to the flattened appearance characteristic of the onset of squamous metaplasia induced by either vitamin A deficiency or carcinogens present in the smoke (Figure 1B). Ultrastructural features were indistinguishable from micrographs of control explants (Figure 2A), except that large amorphous gray vesicles were a conspicuous feature in about a third of the cell (Figure 2B). Furthermore, these explants exhibited prominent mucin gene expression (Figure 2G, b).

Effect of retinoic acid on the squamous phenotype displayed by 10 minute exposed explants

Controls

Normal histological and ultrastructural features (Figures 1A and 2A) were retained when controls were cultured for up to 96 hours in medium with retinoic acid. Mucin gene expression in these controls, however, was prominent in contrast to the low expression in explants prior to incubation. In medium not supplemented with retinoic acid, only the flattened squamous type epithelial sheet was seen and mucin gene expression corresponded to that present prior to incubation (Figure 2G, a). These observations are consistent with the more detailed analyses specifically of retinoid dependent mucin gene expression in rat tracheal cultures (12,15).

Response of 10 minute exposed explants

A mosaic pattern of normal and squamous regions was retained following 48 hour cultivation in medium with or without retinoic acid. The pattern could not be distinguished from that in Figure 1B. Ultrastructural features of the explants at 48 hour of incubation with retinoic acid showed secretory granules with microvilli and occasional cilia in the epithelium (Figue 2C), whereas the explants cultured without retinoic acid indicated flattened epithelium with microvilli and some secretory granules (Figure 2E). Mucin gene expression was prominent in these preparations (Figure 2G, c and H, c). After an additional 48 hour of incubation in medium without retinoic acid, only the flattened pattern characteristic of the squamous phenotype was present (Figure 1C). While the histologic pattern was indistinguishable from the behavior of controls cultured in the same manner, ultrastructural analyses showed pronounced intercellular spaces (Figure 2F) in contrast to the tight cohesive pattern observed in all other explants prior to and following culture. Mucin gene expression in this preparation was drastically reduced (Figure 2H, d). In retinoic acid supplemented medium, there was a remarkable shift to the squamous type histology (Figure 1C) rather than a continuation of the mosaic pattern (Figure 1B) or a reconstitution of the normal mucociliary pseudostratified columnar pattern (Figure 1A). The ultrastructural characteristic of this preparation is shown in Figure 2D, where some cilia and microvilli were still visible. Though the epithelium now exhibited only the flattened morphology characteristic of squamous phenotype, there was continued prominent mucin gene expression (Figure 2G, d).

Response of 10 minute exposed explants to culture in retinoic acid supplemented medium containing either mucin mRNA antisense or sense oligomers

Since prominent mucin gene expression by the squamous epithelium could contribute to obstruction of the smoke damaged airways, the capability of antisense constructs to affect expression was studied with the resulting responses shown in Figures 1D and 4. Mucin gene

expression and secretion were repressed (2.6 and 2.9 folds respectively) by the antisense oligomer (Figure 4C, b) and unexpectedly the epithelium histology shifted back to the columnar pseudostratified architecture (compare Figure 1B to 1D). The sense oligomer affected neither mucin gene expression (Figure 4C, a) nor any change in the histology of the 10 minute exposed explants. Ultrastructural analyses showed the continued prominent appearance of the large amorphous grayish dark vesicles in cultures containing the sense oligomer (Figure 4A), whereas these were no longer detected in cultures containing the antisense oligomer (Figure 4B).

Discussion

In vivo studies have provided a pattern of tissue response and host defense system activities occurring in both model systems (rabbit and sheep) and in burn patients as a result of smoke exposure. Activation of the host defense system results in inflammation associated with the influx of leukocytes, primarily neutrophils, from the circulation in response to the release of chemotactic factors from the irritated tissue. These progressive inflammatory reactions are part of a sequence of changes which occur in the respiratory tract, the most pronounced of which is sloughing of the lumenal surface epithelial sheath that leads to obstruction of the bronchial and tracheal air passage and subsequent deterioration of pulmonary capacity (1-10, 16-18). Though in vivo studies are important in establishing which elements in an animal's complex defense systems are involved in response to an environmental challenge, they cannot distinguish between parallel extrinsic effects, such as the influx of neutrophils on the subsequent sloughing of the epithelium, from those responses intrinsic to the affected respiratory tissue. In the present studies, the results of the 15-20 minute smoke exposures indicate that sloughing of the mucociliary epithelium is a response intrinsic to the irritated respiratory tissue and does not require the participation of extrinsic features such as the influx of leukocytes. The loss of the epithelium was not accompanied by further deterioration of the tissue (i.e., the degeneration of the mucosal and/or cartilagenous layers). The shredding of the epithelium was preceded by a stage noted in 10 minute exposed explants in which the complete integrity of the tracheal tissue architecture (i.e., epithelial layers at the lumenal surface overlying mucosa and cartilagenous layers) was maintained, but there was a shift from the normal pseudostratified columnar architecture, an architecture maintained in control explants, to a mosaic pattern of normal regions and those in which the epithelium was transformed to the flattened appearance characteristic of the onset of the squamous metaplasia. The same transformation stage was previously observed in in vivo vitamin A deficiency (19) and in in vitro cultures where the medium was not supplemented with retinoic acid (20). The squamous metaplasia observed in these studies was reversed by adding vitamin A to the diet or retinoic acid to the culture medium. In contrast, 10 minute smoke exposed explants cultured in medium supplemented with retinoic acid was not effective in reversing the metaplasia, but rather the flattening progressed so that normal regions were detected after 96 hours of incubation. This smoke induced effect apparently was only a change in the epithelial architecture, presumably an effect on either the cytoskeleton or adhesion processes, since it was not accompanied by changes in other characteristics associated with transformation from the mucociliary phenotype to the squamous state, namely an increased expression of mucin gene, the absence of secretory granules, and the appearance of keratin bundles. These same 10 minute smoke exposed explants, when cultured in the medium without retinoic acid, showed prominent intercellular spaces, suggesting

degeneration of the cellular architecture involving cellular adhesion. Loss of cellular adhesion indicates that retinoic acid is required for maintaining adhesive cell to cell contacts following smoke exposure.

Reversion to the normal pattern was attained when the explants were transvected with antisense 18-mer mucin oligomer. Significant suppression of the secretion of mucin was also achieved with antisense oligonucleotide. Screen of the gene bank and EMBL data bases for RNAs and DNAs revealed sequence homology to versican, a proteoglycan component, which is known to affect cell adhesion (21) and no other cellular cytoskeleton proteins. This study does not, however, address any relationship between the effects of mucin antisense and versican molecules. The basis for the antisense action remains to be determined. However, since the sense oligomer had no corrective effects, the antisense oligomer is very likely reacting with a mRNA that is involved in the reversal of squamous to normal pseudostratified columnar phenotype.

The use of antisense oligomer to modulate gene expression and correct genetic disorders, both negative and recessive, is being used now to find alternate approaches for therapeutic intervention in disease processes (22-26). The studies presented in this report demonstrate that mucin antisense oligomer has positive effects in maintaining a healthy epithelium and inhibiting mucus oversecretion when the trachea, exposed to noxious agents such as smoke, is cultured in a synthetic medium supplemented with retinoic acid. It will be investigated further whether development of a therapy based on this observation can be used as an alternate approach in the management of various respiratory diseases.

7. Conclusions

In conclusion, rabbit tracheal explants, after exposure to smoke from burning pine wood, showed inflammation in the epithelium with changes in histology and ultrastructure as well as changes in the expression of mucin gene. Retinoic acid and a mucin antisense oligomer were found to inhibit mucin gene expression and secretion, at the same time keeping the epithelium normal. Maintenance of cellular adhesion of the epithelium, after exposure to smoke, was dependent on the presence of retinoic acid in the culture medium. Thus, combined therapy of retinoids and mucin antisense oligomer may have some clinical implication in stabilizing as well as inhibiting mucus hypersecretion in tracheobronchial epithelium of people, including soldiers in combat situation, exposed to toxic substances like smoke.

We are now engaged in developing a liposome-based delivery system which can be used to deliver the two components, cited above, in in vivo situation. We will examine the long-term effect of smoke exposure on tracheal epithelium of the model system, such as rabbit, and the process of the stabilization of the epithelium and inhibition of mucus oversecretion by the use of retinoids and mucin antisense oligomer.

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9. **Appendices**

a. Reprints of the first paper published in **Inflammation** are attached with this report. Manna, B., Ashbaugh. P., and Bhattacharyya, SN. 1995. Retinoic acid-regulated cellular differentiation and mucin gene expression in isolated rabbit tracheal epithelial cell in culture. Inflammation **19**: 489-502. Attached.

b. The report, as presnted here, has been accepted for publication in **Inflammation.** Bhattacharyya, SN., Manna, B., Smiley, R., Ashbaugh, P., Coutinho, R., and Kaufman, B. 1997. Smoke induced inhalation injury: Effects of retinoic acid and antisense oligodeoxy-nucleotide on stability and differentiated state of the mucociliary epithelium. Inflammation. In press.

c. An abstract of the present findings was presented to "Gene Therapy for Acquired Diseases", held at Vanderbilt University Medical Center, Nashville, TN., on October 19-21, 1995. Bhattacharyya, SN., and Manna, B. 1995. Regulation of mucin gene expression in rabbit trachea. Attached.

Figure 1. Light microscopic examination (H & E) of rabbit tracheal explants exposed to smoke for 10 minutes and cultured in a synthetic medium. A: Control (without smoke) showing pseudostratified columnar epithelium (x100). B: Explants exposed to smoke for 10 minutes showing part columnar and part flattened epithelium (x100). Similar pattern was noticed when smoke exposed explants were cultured for 48 hours in the medium with or without retinoic acid. C: Smoke exposed explants cultured for 96 hours in the medium without retinoic acid showing flattened epithelium (squamous phenotype, x100). Similar phenomenon was noticed when smoke exposed explants were cultured in the medium with retinoic acid. D: Smoke exposed explants cultured for 96 hours in retinoic acid supplemented medium with mucin antisense oligomer showing pseudostratified columnar wpithelium (x100).

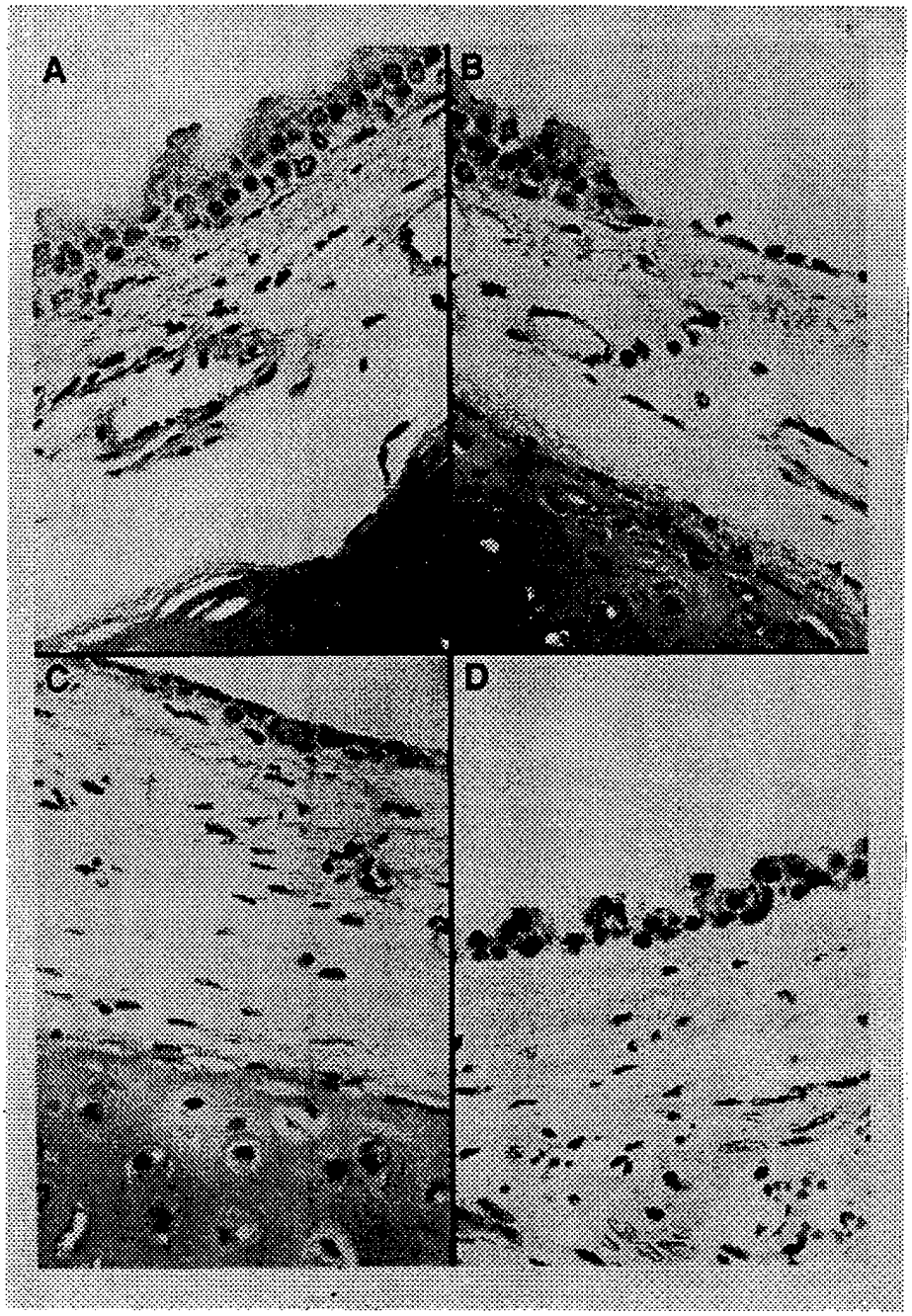


Figure 2. Transmission electron microscopy and dot blot hybridization of total RNA(3 μg) from smoke exposed (10 minutes) explants cultured for 48 and 96 hours in a synthetic medium with and without retinoic acid. Ultrastructure: A: Control showing normal features containing secretory vesicles (V), cilia and microvilli. B: Explants after smoke exposure for 10 minutes showing large secretory granules (S), desmosomes, cilia (broken arrow) and micovilli. C: Smoke exposed explants cultured for 48 hours in medium with retinoic acid showing normal features containing desmosomes, microvilli and cilia (broken arrow). D; smoke exposed explants cultured for 96 hours in medium with retinoic acid showing partial columnar epithelium with few secretory vesicles and micrvilli. E: Smoke exposed explants cultured for 48 hours in medium without retinoic acid showing flattened epithelium (squamous phenotype) containing few secretory granules (S) and micovilli (arrow). F: Smoke exposed explants cultured for 96 hours in medium without retinoic acid showing partial flattening of epithelium with loss of cellular adhesion though cilia, microvilli and intercelluar junctions were still visible. N: nucleus. Magnification x 4500. Dot blot hybridization: G: a:Control, b: 10 minute exposure, c: smoke exposed explants cultured for 48 hours in medium with retinoic acid, d: smoke exposed explants cultured for 96 hours with retinoic acid. H: a: Control not applied, b: 10 minute exposure, c: smoke exposed explants cultured for 48 hours without retinoic acid, d: smoke exposed explants cultured for 96 hours without retinoic acid. G-H, hybridized with mucin probe. I: a-d, β-actin level. J: a-d, type I keratin level.

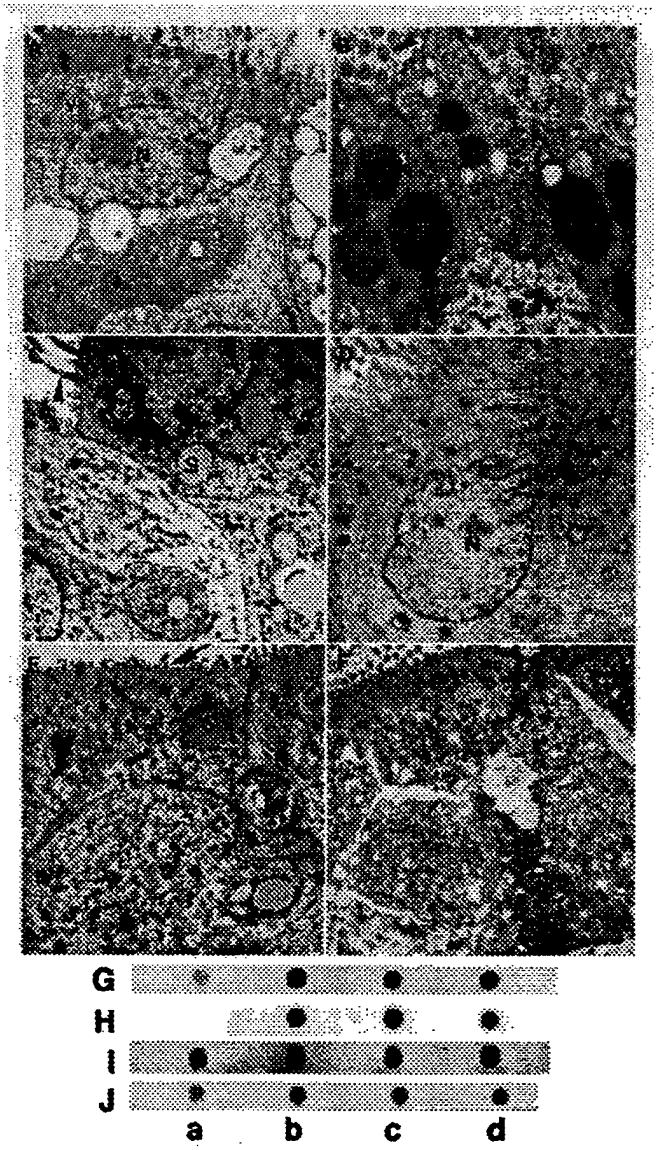


Figure 3. Transmission electron microscopy of rabbit tracheal explants exposed to smoke for 20 minutes showing extreme degeneration (sloughing) of epithelium with numerous vacuoles. The explants could not be cultured for lack of many live cells. Magnification x 10,000.

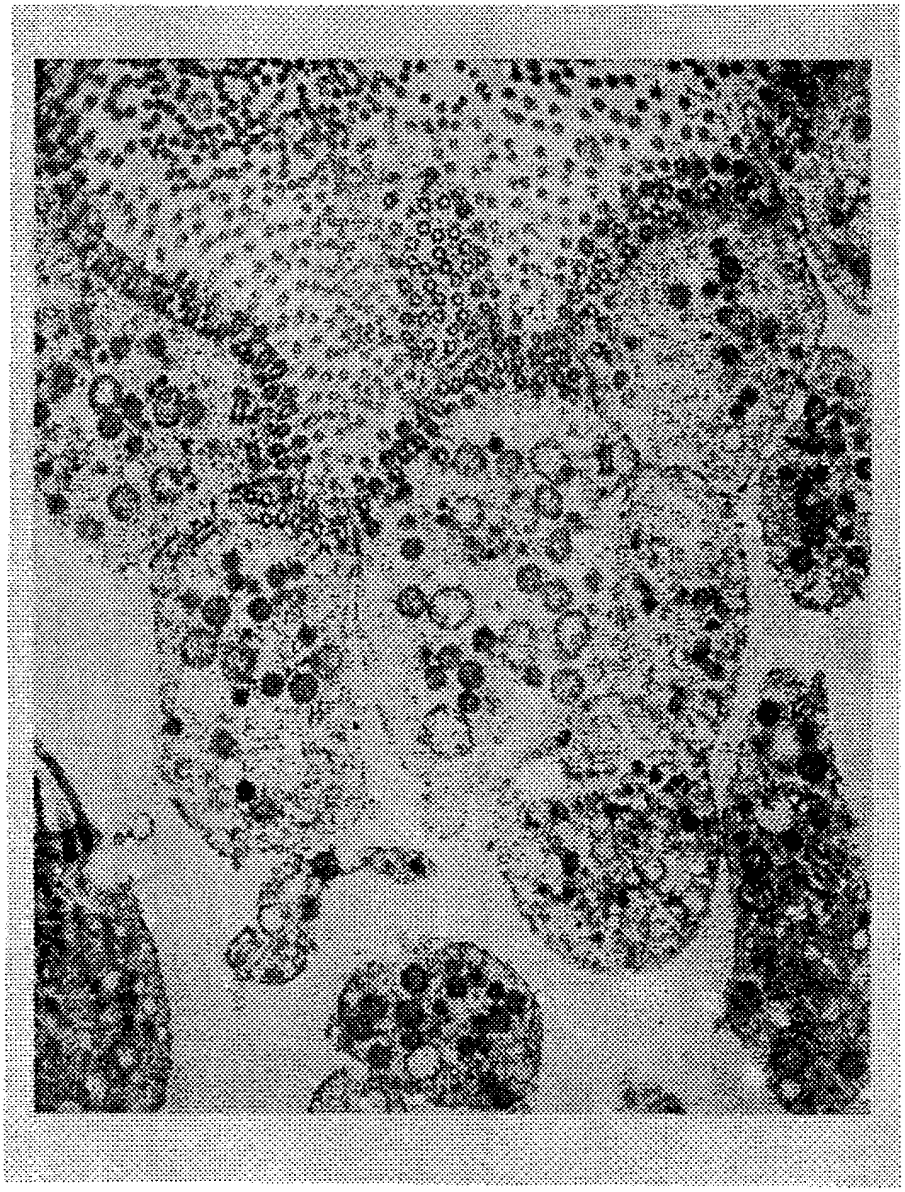
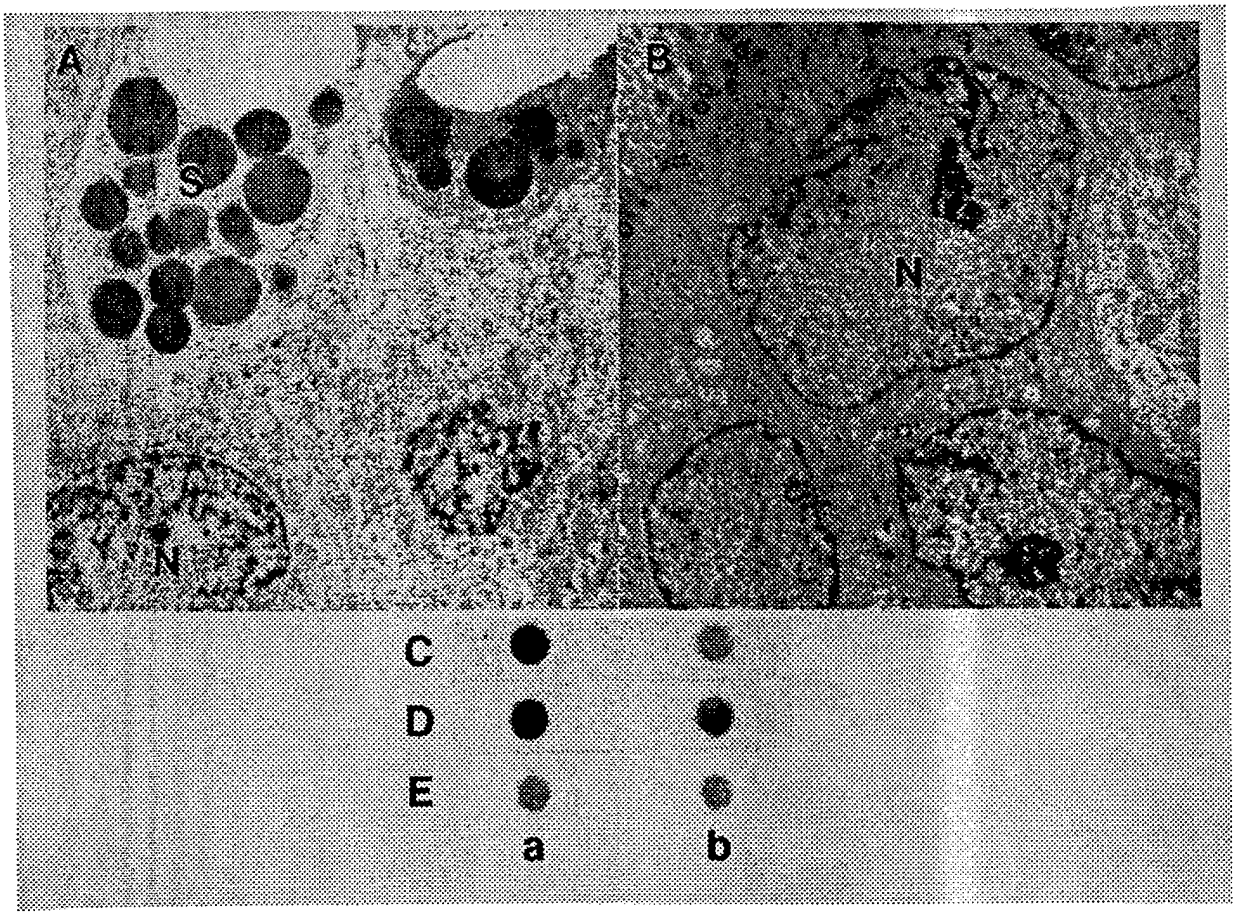


Figure 4. Transmission electron microscopy and dot blot hybridization of total RNA (3 μg) from smoke exposed (10 minutes) rabbit tracheal explants cultured for 96 hours in retinoic acid supplemented medium with mucin sense (A) and antisense (B) olgomers. A: Explants with sense oligomer showing normal epithelium with numerous secretory granules. B: Explants with antisense oligomer showing normal epithelium with cilia, microvilli and desmosomes without secretory granules. N: nuleus. Magnification x 11,000. Dot blot hybridization: C: a: Sense, b: antisense (hybridized with mucin probe). D and E: a-b, β-actin and type I keratin levels in the same preparation.



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RETINOIC ACID-REGULATED CELLULAR DIFFERENTIATION AND MUCIN GENE EXPRESSION IN ISOLATED RABBIT TRACHEAL EPITHELIAL CELLS IN CULTURE[1]

BRIGITTA MANNA,[2] PATRICK ASHBAUGH,[3] and SAMBHU N. BHATTACHARYYA[2]

[2]Department of Clinical Investigation
[3]Electronmicroscopy Section
William Beaumont Army Medical Center
El Paso, Texas 79920

Abstract—Rabbit tracheal epithelial cells were cultured in a serum-free and hormone-supplemented medium with and without retinoic acid. The cells showed time-dependent mucin gene expression when cultured in the medium with retinoic acid. In the absence of retinoic acid, however, mucin mRNA was barely detectable in the cells. When retinoic acid was added back to the medium, the mucin message was prominent again. Actinomycin D and cycloheximide did not inhibit mucin gene expression. The mucin message was slightly elevated by cAMP agonists. A mucin antisense oligomer inhibited the retinoic acid-induced mucin mRNA expression and secretion, thus offering an alternate approach in the management of mucus hypersecretion in upper airway respiratory diseases such as chronic bronchitis, asthma, and cystic fibrosis.

INTRODUCTION

Bronchial mucus glycoproteins (mucins) are localized on the apical surface of the mucus membrane, where they function in the mucociliator–escalator systems that protect the airways from microscopic particles and infections. Excessive accumulation of mucus is one of the major contributors to airway obstruction in people suffering from upper airway respiratory diseases, such as asthma, chronic bronchitis, and cystic fibrosis. The precise mechanism(s) involved in the accumulation of mucus in these diseases is not known, but all evidence

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points to overproduction rather than defective clearance by the mucocilary systems.

It has been reported (1–3) that tracheal epithelium can be maintained in a serum-free and hormone-supplemented medium containing retinoids (vitamin A) for several weeks; cultured cells were found to secrete mucins into the medium, as measured by the incorporation of radiolabeled sugars. Previously, we observed (4) that rabbit tracheal epithelial cells, grown in a serum-free and hormone-supplemented medium, expressed mucin gene when total RNA isolated from the cells was hybridized with a 30-mer oligonucleotide probe derived from a rat intenstine peptide tandem repeat sequence, TTTPDVTTTP, RMUC 176 (5). However, in the absence of retinoic acid in the culture medium, the cells did not grow or produce mucin. Recently, we observed (6, 7) that the expression of mucin mRNA in rat tracheal explants at various times of growth in a serum-free and hormone-supplemented medium was strongly induced by retinoic acid. In the absence of retinoic acid, the mucin message in the tracheal organ culture decreased considerably. Addition of retinoic acid to the deficient medium restored the mucin message back to normal. Addition of various pharmacologic agents also had some marginal effect on mucin mRNA expression in rat tracheal organ culture grown with and without retinoic acid. However, the effect of these reagents on mucin mRNA expression in an isolated tracheal epithelial cell culture system has not been reported.

Recently, there has been intense interest in developing antisense agents for sequence-specific inhibition of gene expression (7–10) and using them as therapeutic agents. Thus, viral as well as cancer message expression has been successfully inhibited by antisense oligomers. In most of these experiments, the antisense oligomer has been chemically modified, and the most frequent use was that of phosphorothioate derivative. The same derivative of an antisense oligomer has recently been used in the treatment of acute myeloblastic leukemia (11).

In view of the above findings, we have examined the control as well as stability of mucin mRNA expression in isolated rabbit tracheal epithelial cells in culture in a serum-free and hormone-supplemented medium with and without retinoic acid and the effect of an antisense oligodeoxynucleotide in inhibiting this retinoic acid-induced mucin gene expression.

MATERIALS AND METHODS

All chemicals were of the highest quality available and obtained from various sources as described before (1, 4, 6, 7).

Cell Culture

Airway epithelial cells were isolated from rabbit trachea (New Zealand white rabbit, Hazelton Research Laboratories, Denver, Pennsylvania), and cultured on collagen-coated dishes in a serum-free and hormone-supplemented medium according to the procedures described prviously (1, 4, 6, 7). Cells were incubated at 37°C in a humidifed incubator containing air-CO_2 (19:1), and the medium was changed every two days. The epithelial nature of the cells containing secretory granules, microvilli, tonofilaments, and desmosomes has been established in our laboratory by electron microscopy (4).

Isolation of RNA

Total RNA was isolated from cells (2.0×10^6) using Clontech's RNA isolation kit, which utilizes a guanidium thiocyanate-phenol-chloroform single-step extraction procedure, as described previously (4, 6, 7, 12). The extracted RNA was precipitated with 3 M sodium acetate and absolute alcohol and kept at −70°C until needed.

Northern and Dot Blot Hybridization

RNA was collected by centrifugation at 5000*g*, dried under vacuum, and rehydrated with a minimum volume of TE buffer and RNAase inhibitor, as described before (4, 6, 7). An aliquot of RNA preparation (5–10 μg) was run on a formaldehyde-agarose (1%) gel in 1 × Mops buffer (6). The gel was stained with ethidium bromide. RNA was transferred to hybond membrane from the gel for Northern hybridization. The membrane was dried under vacuum at 80°C for 2 h.

A direct transfer of RNA (0.5–1.5 μg) to spots on the Hybond membrane was made in a dot blot apparatus and the membrane was dried as described above. The membrane was hybridized with an E-linked antisense 30-mer oligonucleotide to rat intestine mucin protein tandem repeat sequence, RMUC 176, TTTPDVTTTP, (5′-X AGG GGT GGT GGT CAC ATC AGG AGT GGT GGT-3′, X = amine) (5)—this procedure has been described in detail elsewhere (4, 6, 7). After hybridization, the membrane was stained with Lumi-Phos solution, placed between two acetate sheets, and exposed to Kodak XAR-5 film for 3 h at 37°C. All experiments were performed with the same concentration (1.0 nM) of the probe under identical conditions.

The membrane was also hybridized with an E-linked antisense 30-base oligonucleotide to mouse type I keratin protein sequence, GGDQSSKGPR (5′-X TGG TCC TTT AGA TGA TTG GTC GCC GCC ACC-3′, X = amine) (13), as described before (6, 7).

The following phosphorothioate derivatives of oligonucleotides were used in these studies: sense oligo—5′-ACCACCACTCCTGACGTC-3′ to rat intestine mucin cDNA sequence (5) (control); and antisense oligo—5′-AGGGGTGGTGGTCACATC-3′ to rat intestine mucin cDNA sequence (5).

Effect of Retinoic Acid, Actinomycin D, Cycloheximide, cAMP Agonists, and Antisense Oligomer

Retinoic Acid. Time-dependent expression of the mucin message with and without retinoic acid (0.1 μM) was investigated by incubating the rabbit tracheal epithelial cells (2.0×10^6) in 3.0–4.0 ml of culture medium for 0, 48, 96, 120, and 144 h under the conditions described before (4, 6, 7). In order to determine the effect of retinoic acid on mucin mRNA content in the cells grown in medium without retinoic acid, the cells were initially maintained in a retinoic acid-deficient medium for 96 h before addition of this reagent, and the incubation continued for another 96 and 144 h. Duplicates and controls were run under the same conditions.

Actinomycin D and Cycloheximide. The effect of actinomycin D (20 μg/ml) and cycloheximide (20 μg/ml) on the expression of the mucin message in these cells grown in the presence of retinoic acid was examined by adding these reagents at 96 h of growth and continuing incubation for additional 12-, 24-, 48-, and 96-h periods of time. Duplicates and controls were run under the same conditions.

cAMP Agonists. In order to determine the effect of cAMP agonists, forskoline (100 μM), PGE_2 (100 μM), bt_2cAMP (100 μM), and the calcium ionophore A23187 (100 μM), on mucin gene xpression in these cells, the cells were grown for 96 h before addition of these reagents to the medium and the incubation continued for another 48 h. Duplicates and controls were run under the same conditions.

Sense and Antisense Oligonucleotides. The cells (2.0×10^6) were grown for 120 h before addition of 10 μCi of [6-^{3}H]glucosamine (4), sense and antisense oligomers (0.53 nmol/ml), and the incubation continued for another 48 h. After the reaction, the cells were removed from the medium by centrifugation. Duplicates were run under the same conditions.

Total RNA was isolated from the cells and hybridized with the respective oligonucleotide probe, as described above. The density of the signal was measured by the BioRad densitometer.

The medium was dialyzed against water at 4°C overnight with two changes and lyophilized. The lyophilized material was digested with hyaluronidase and analyzed for mucin by Sepharose 2B chromatography and gel electrophoresis as described in detail elsewhere (4). Radioactivity was determined in a Beckman LS 3801 counter.

Phase-Contrast and Electron Microscopy.

The cells at different stages of growth were examined with a phase-contrast microscope.

The cells prepared for electron microscopy were fixed in 3.5% (w/v) glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections of the sample were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope (4, 6, 7).

RESULTS

The changes in the morphology of rabbit tracheal epithelial cells were examined by phase-contrast microscopy at 48, 96, and 144 h of growth in a medium with and without retinoic acid (Figure 1 A–F). As is seen in this figure

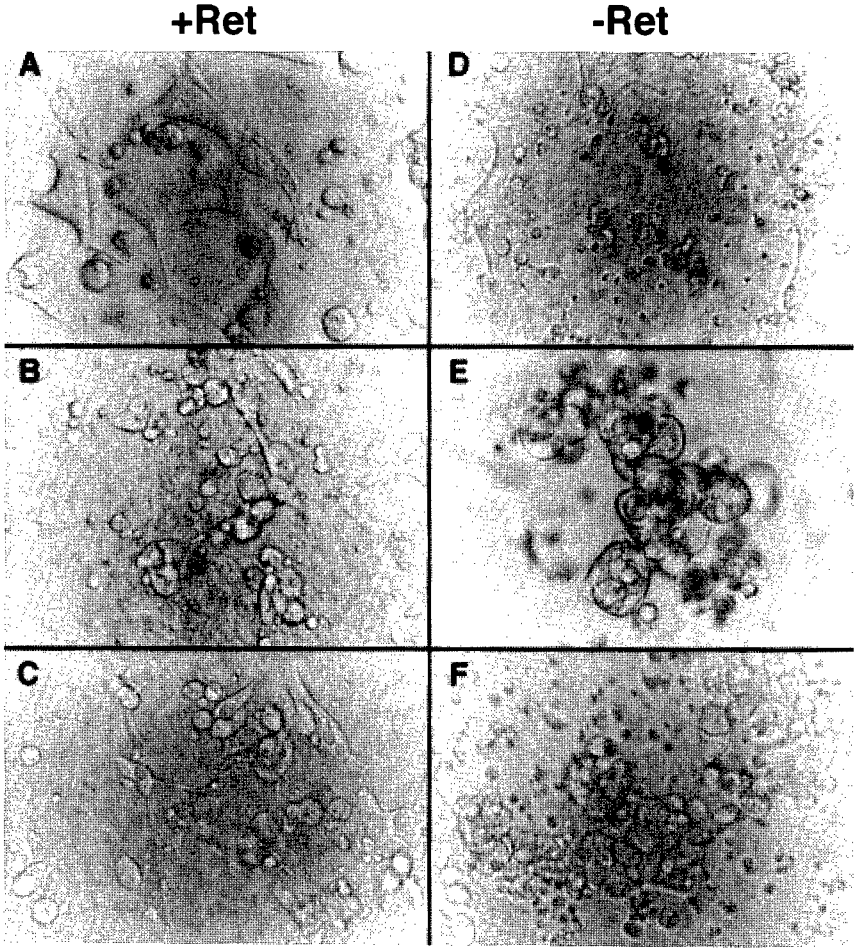

Fig. 1. Phase-contrast micrograph of rabbit tracheal epithelial cells at 48, 96, and 144 h of growth in a serum-free and hormone-supplemented culture medium with and without retinoic acid. The cells were cultured as described in Materials and Methods. A, B, C: +retinoic acid; D, E, F: −retinoic acid. Magnification ×160.

and as also observed by several investigators in tracheal epithelial cells from other animals (14–16), the difference in morphology of the cells grown with and without the presence of retinoic acid in the medium is quite striking. Retinoic acid-sufficient cells had a tendency to form monolayers with the passage of time, whereas deficient cells showed changes towards squamous differentiation. At a later stage of growth, the retinoic acid-deficient cells tend to detach themselves

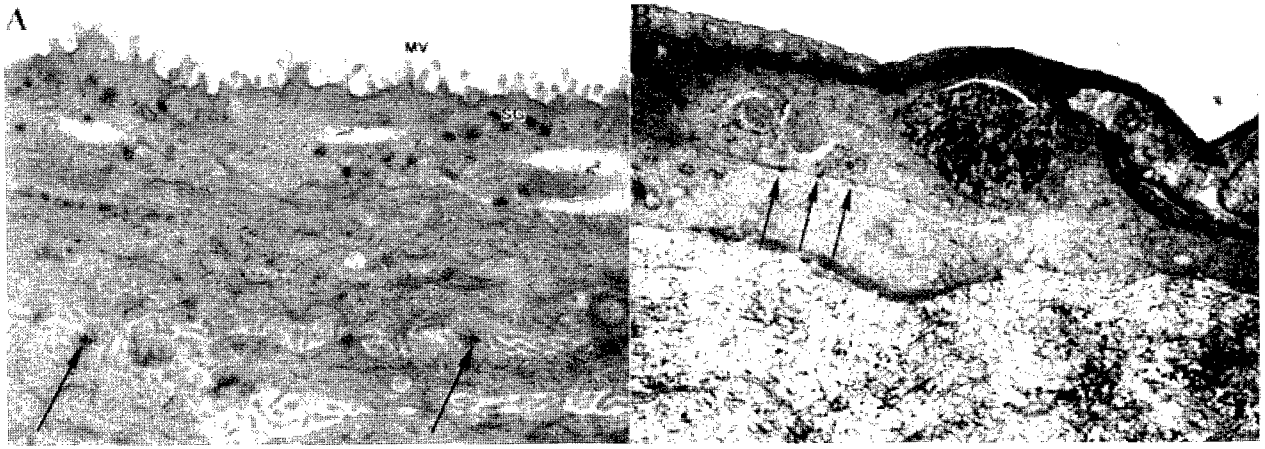

Fig. 2. Transmission electron microscopy of rabbit tracheal epithelial cells grown with and without retinoic acid. (A) (+retinoic acid): SG, secretory granules; MV, microvilli; arrow, desmosomes. (B) (−retinoic acid): arrow, desmosomes. Note the stratified epithelial layer of the cells grown without retinoic acid and in absence of secretory granules. Magnification ×13,000.

from the collagen layer. Transmission electron microscopic studies of the cells at 144 h of growth in a medium containing retinoic acid indicated that the cells contained tonofilament bundles and were joined by desmosomes. Microvilli and occassional secretory granules were also observed (Figure 2A). In the absence of retinoic acid, however, the epithelial layers were stratified with development of squamous metaplasia. No secretory granules were observed (Figure 2B). This change in cell morphology was associated with a change in mucin gene expression. Dot blot hybridization using an E-linked rat intestine mucin tandem repeat

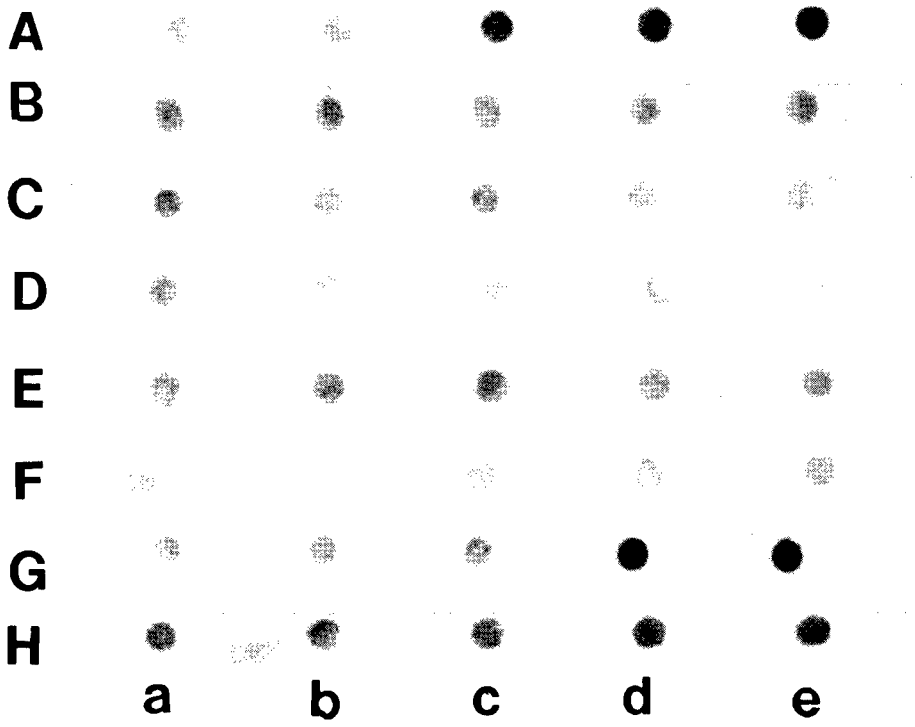

Fig. 3. Dot blot hybridization of total RNA (1.2 μg) from rabbit tracheal epithelial cells at different times of growth in a serum-free and hormone-supplemented medium with and without retinoic acid. Hybridization with E-linked rat intestine mucin probe was performed as described in the experimental section. (A) a–e (mucin probe), total RNA from culture (+retinoic acid) at 0, 48, 96, 120, and 144 h of growth; (B) a–e, same as A except hybridized with β-actin probe; (C) a–e, same as A except hybridized with type I keratin probe; (D) a–e (mucin probe), total RNA from culture (−retinoic acid) at 0, 48, 96, 120, and 144 h of growth; (E) a–e, same as D except hybridized with β-actin probe; (F) a–e, same as D except hybridized with type I keratin probe; (G) a–e, same as D except that retinoic acid was added to the medium at 96 h of growth (c) and the culture was allowed to grow for another 96 and 144 h (d, e); (H) a–e, same as G except hybridized with β-actin probe.

probe, RMUC 176 (5), to total RNA from the cells grown at different periods of time showed that the mucin message, after expressing at a lower level at 0 and 48 h (Figure 3A, a–b), showed a stronger signal from 96 h to all subsequent time points (Figure 3A, c–e). The expression of β-actin mRNA in these preparations is shown in Figure 3B, a–e. Type I keratin message showed a positive signal in all the preparations (Figure 3C, a–e). In the absence of retinoic acid, the mucin message was considerably reduced with the progression of time (Figure 3D, a–e), whereas the expression of β-actin mRNA remained steady (Figure 3E, a–e). The hybridizational signal for type I keratin message was evident in these preparations (Figure 3F, a–e). Addition of retinoic acid to the latter culture at 96 h of growth resulted in the recovery of mucin message within 96 h of additional incubation (Figure 3G, c–e). The level of β-actin message in these preparations is shown in Figure 3H, a–e.

Addition of actinomycin D (20 μg/ml), an inhibitor of transcription, to the culture at 96 h of growth in the medium containing retinoic acid did not result

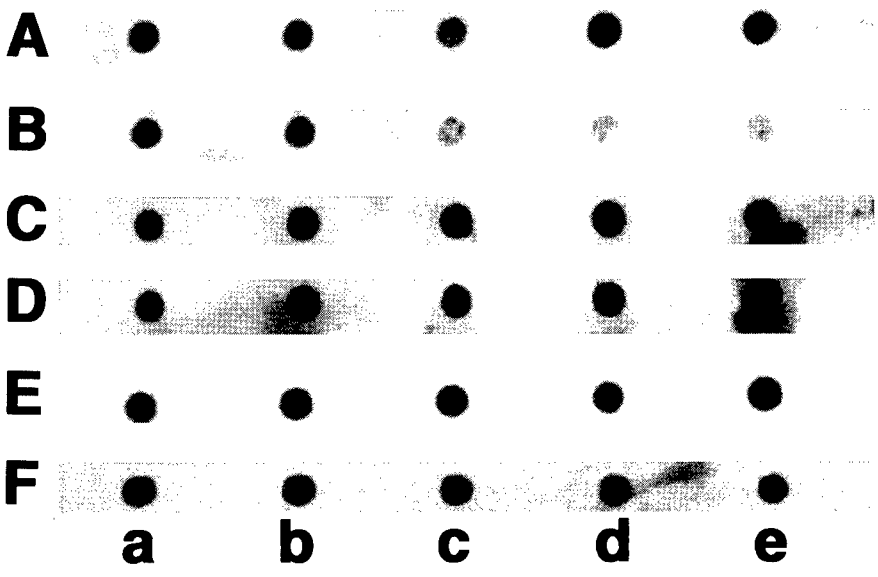

Fig. 4. Effect of actinomycin D (20 μg/ml), cycloheximide (20 μg/ml), cAMP agonists (100 μM), and calcium ionophore A23187 (100 μM) on mucin mRNA from rabbit tracheal epithelial cells grown in a medium with retinoic acid. The cells were grown for 96 h (control) before addition of the reagents, and the incubation continued for the specified period of time. Total RNA (1.5 μg) was hybridized with E-linked rat intestine mucin probe as described in the experimental section. (A) a–e (actinomycin D), control, 12, 24, 48, and 96 h; (B) same as A except hybridized with β-actin probe; (C) a–e (cycloheximide), control, 12, 24, 48, and 96 h. (D) a–e, same as C except hybridized with β-actin probe; (E) a (control), b (forskoline), c (PGE_2), d (bt_2cAMP), and e (calcium ionophore A23187); (F) a–e, same as E except hybridized with β-actin probe.

in the reduction of the mucin message (Figure 4A, a–e) and was similar to the pattern shown in Figure 3A, a–e, whereas β-actin mRNA expression decreased considerably (Figure 4B, a–e). The inhibitor of translation, cycloheximide (20 μg/ml), slightly increased the mucin message (Figure 4C, a–e). The level of β-actin mRNA in these preparations is shown in Figure 4D, a–e. Incubation of the cells at 96 h of growth with activators of the cAMP pathway, forskoline, PGE_2, and bt_2cAMP, and calcium ionophore A23187 resulted in a slight increase in the expression of the mucin message (Figure 4E, a–e). The expression of β-actin message in these preparations is shown in Figure 4F, a–e.

Agarose gel (1%) electrophoresis of the total RNA from rabbit tracheal epithelial cells at 144 h of growth showed a typical gel profile of 28S and 18S RNA in the preparation (Figure 5A). Northern hybridization of the RNA prep-

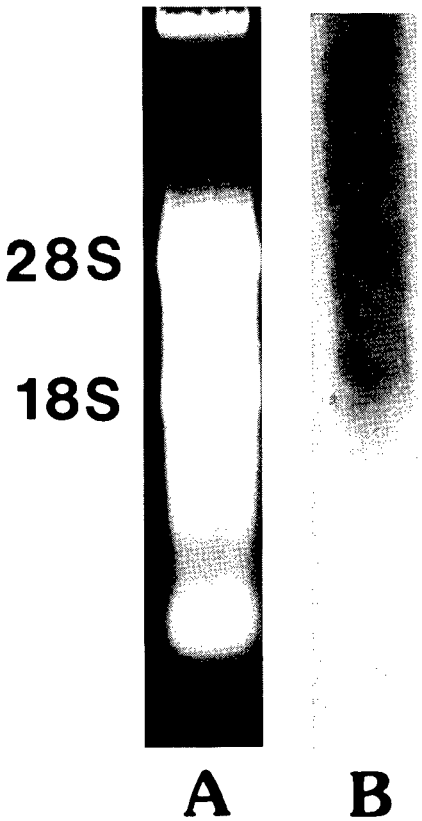

Fig. 5. (A) Formaldehyde-agarose gel (1%) electrophoresis of total RNA (10 μg) from rabbit tracheal epithelial cells at 144 h of growth in a serum-free and hormone-supplemented medium with retinoic acid. The gel was stained with ethidium bromide. (B) Northern hybridization of total RNA after transfer to the hybond membrane and probed with E-linked mucin probe.

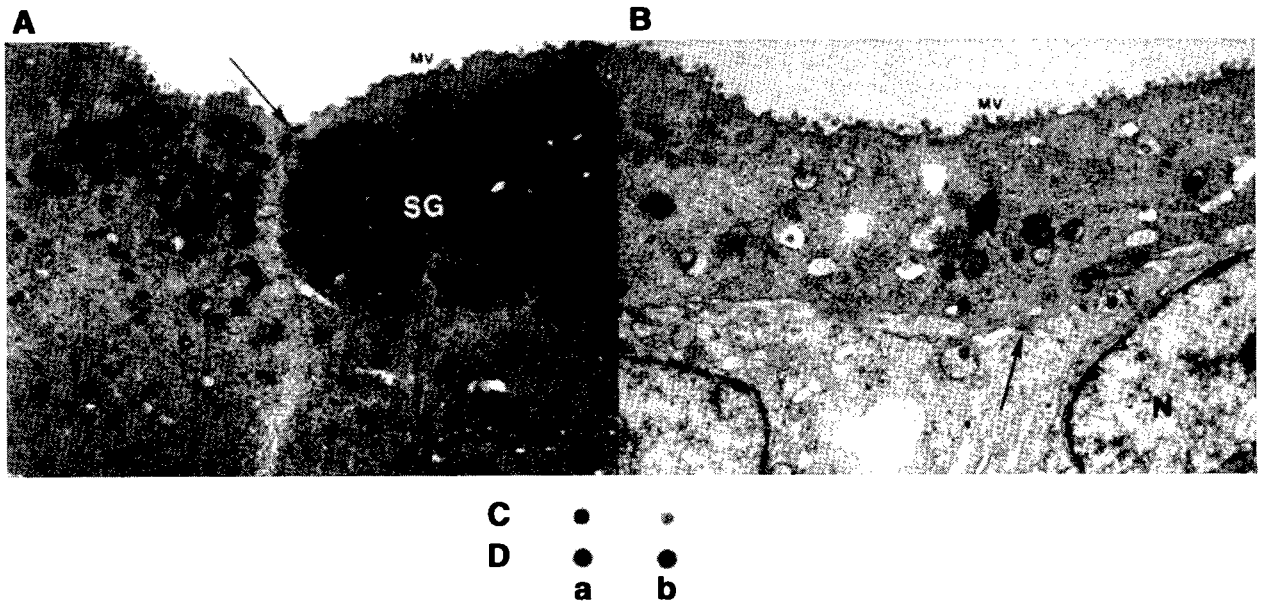

Fig. 6. Transmission electron microscopy of rabbit tracheal epithelial cells treated with sense (A) and antisense (B) oligonucleotides. The cells were grown in medium with retinoic acid as described in the methods section. (A) MV, microvilli; SG, secretory granules; arrow, junctional complexes; N, nucleus. Magnification ×14,000. (B) MV, microvilli; arrow, desmosome. No secretory granules were noted. Magnification ×13,000. (C) a–b, dot blot hybridization of total RNA (1.5 μg) from cells treated with sense (a) and antisense (b); (D) a–b, same as C except hybridized with β-actin probe.

aration resulted in a diffuse pattern with a molecular weight ranging from 2.0 kb to >9.5 kb (Figure 5B). Such a polydisperse signal of mucin mRNA on Northern analysis has been observed by others before (17–20). The reason for this polydispersion is not known.

Transmission electron microscopy showed that the cells grown in medium containing retinoic acid and the phosphorothioate derivative of the sense oligomer were covered with surface microvilli and joined by small desmosomes and apical junctional complexes (Figure 6A). In addition to numerous secretory granules, the cytoplasm contained Golgi complexes, mitochondria, and segments of rought endoplasmic reticulum. In the presence of antisense oligomer, the cells displayed surface microvilli and small desmosomes, but no secretory granules (Figure 6B). This change in cell morphology was associated with a change in mucin gene expression and secretion in these cells. Addition of the antisense oligomer to the cells resulted in an approximate 2.3-fold decrease in the expression of mucin mRNA when compared to the effect of the sense (control) oligomer (spot density = sense, 9.35: antisense, 4.15) (Figure 6C, a–b). Figure 6D, a–b indicates the β-actin message expression in these preparations. The decrease in the expression of mucin mRNA was followed by 2.4-fold drop in the secretion of mucin into the medium [radioactivity (cpm/ml): sense, 8940, antisense, 3754).

DISCUSSION

The present study, demonstrating that retinoic acid stimulated the growth of isolated rabbit tracheal epithelial cells in a serum-free and hormone-supplemented culture medium and caused up-regulation of mucin message, is an extension of our previous studies (6, 7), in which we noted that retinoic acid was also required for optimal mucin gene expression in rat tracheal explants grown in the same culture medium. In the absence of retinoic acid, rabbit tracheal epithelial cells did not grow normally, nor did they express mucin gene strongly. Instead, the entire epithelum was stratified with an additional change towards squamous metaplasia. However, the mucin message recovered when retinoic acid was reintroduced to the retinoic acid-deficient medium. Thus, the mucin gene in rabbit tracheal epithelial cells in culture seems to be transcriptionally activated by retinoic acid, and the mucin mRNA expression is strongly associated with differentiation and proliferation of epithelial cells.

The results presented in this report and in our earlier studies (4, 6, 7) regarding the effect of retinoic acid in the enhancement of mucin gene expression are in agreement with those of Jany and Basbaum (21), who suggested that induction of the mucin message is the primary event that leads to increased

synthesis and secretion of mucins in rat trachea exposed to SO_2. Retinoid-dependent gene regulation has been observed previously in squamous differentiation of tracheal epithelial cells (22).

The persistence of mucin mRNA at high levels in the presence of the transcription inhibitor, actinomycin D, indicates that mucin transcripts in these cells are highly stable molecules. The inhibitor of translation, cycloheximide, did not block the on-going protein synthesis in the cells. Stabilizations of neurofilament mRNA in primary sensory neurons, vitellogenin mRNA by estrogen in primary liver cells, and transferrin receptor mRNA by chelation of iron have been found to occur in the presence of both actinomycin D and cycloheximide. It has been suggested that the process could be mediated by short-lived factor(s) that protects the transcripts from destabilization due to the presence of hormones or ligands (23–25). Such a mechanism involving the retinoic acid receptor complex or a cascade of regulatory proteins in tracheal epithelial cells may exist to protect the mucin message from destabilization. Alternatively, the mucin transcripts could be indirectly stabilized by factor(s) that regulate both cAMP pathway as well as those involved in mucin synthesis (2, 4, 26, 27). However, no effects comparable to those seen in the presence of retinoic acid were observed upon addition of either forskoline, PGE_2, bt_2cAMP, or calcium ionophore A23187 to these primary tracheal epithelial cell lines.

The study as presented here clearly indicates that retinoic acid regulates mucin gene expression in isolated rabbit tracheal epithelial cells cultured in a serum-free and hormone-supplemented medium and that this expression is associated with differentiation and proliferation of these cells. This retinoic acid-induced mucin gene expression and secretion also can be inhibited by mucin antisense oligomer. The use of an antisense oligomer to regulate gene expression is fast becoming an alternative approach for therapeutic intervention into disease processes such as cancer and viral infection (7–11). The present study can be extended further to examine whether antisense therapy can be used in the management of hypersecretion of mucus in upper airway respiratory diseases such as chronic bronchitis, asthma, and cystic fibrosis.

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FIRST AUTHOR (Please type information)

Name Dr. Sambhu N Bhattacharyya

Affiliation Department of Clinical Investigation #55

Address William Beaumont Army Medical Center

El Paso, Texas 79920

Telephone (915) 569-2233/1041 Fax (915) 569-1500

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REGULATION OF MUCIN GENE EXPRESSION IN RABBIT TRACHEA

S.N. Bhattacharyya and B. Manna, Department of Clinical Investigation, William Beaumont Army Medical Center, El Paso, Texas 79920

Rabbit tracheal epithelial cells were cultured in a serum-free and hormone-supplemented medium with and without retinoic acid. The cells showed time-dependent mucin gene expression when cultured in the medium with retinoic acid. In the absence of retinoic acid, the mucin mRNA was barely detectable in the cells. When retinoic acid was added back to the medium, the mucin message was prominent again. Actinomycin D had no effect on mucin mRNA expression in cultures grown with retinoic acid. Cycloheximide had also no effect on mucin mRNA in retinoic acid sufficient cultures. cAMP agonists had some marginal effects on the mucin mRNA expression. A mucin antisense oligomer inhibited the mucin mRNA expression and secretion. The results from our studies suggest that differentiation and hence the induction of mucin gene expression in rabbit tracheal epithelial cells by retinoic acid contribute to regulation of the synthesis of these important components. These findings have important implications in various respiratory diseases, such as asthma, cystic fibrosis, and chronic bronchitis, which are characterized by oversecretion of mucus.

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